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# Genotyping of *Toxoplasma gondii* Isolates with 15 Microsatellite Markers in a Single Multiplex PCR Assay[▽]

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**We developed an easy-to-use method for genotyping *Toxoplasma gondii* isolates in a single multiplex PCR assay with 15 microsatellite markers. This method was validated by testing 26 reference isolates that had been characterized with other sets of markers.**

The commonly used multilocus methods for genotyping *Toxoplasma gondii* strains employ PCR-restriction fragment length polymorphism (PCR-RFLP) analysis at 10 markers (16), sequencing at 8 introns of 5 loci (14), and length polymorphism analysis of 5 microsatellite (MS) markers (4). Advantages and disadvantages of these different markers have been reviewed elsewhere (16). Based on our experience within the *Toxoplasma* Biological Resource Center (BRC ToxoBS) and the National Reference Center for Toxoplasmosis, it appears that screening of a large number of clinical isolates for *T. gondii* strain typing should rely on an easy-to-use method with two different levels of discrimination. The first step of discrimination is the performed at the typing level: that is, employing the ability of markers to distinguish the major clonal lineages from atypical strains. In areas with a marked clonal population structure such as Europe, the genetic screening of clinical isolates should rapidly identify basic type II or III strains, which represent the majority of isolates, and atypical strains, which are the exception to the rule (3, 11). In this context, the identification of atypical strains is of clinical and epidemiological importance because atypical strains are usually associated with severe disease outcomes and contamination of individuals by non-European strains either during residence abroad or after consumption of imported meat (5, 9, 10). The second level of discrimination is the fingerprinting level, representing a high degree of discriminatory power for differentiating closely related strains belonging to the same lineage. This high-resolution analysis is required for identifying laboratory contaminations for diagnosis issues and for establishing a common source of infection among different infected individuals in an outbreak (7, 8).

Microsatellite (MS) sequences are tandem repeats of short (1 to 6 bp) DNA motifs that are ubiquitous in eukaryotic genomes and undergo length changes due to insertion or deletion of one or multiple repeat units. The most commonly proposed mutation mechanism for MS sequences is strand slippage, occurring predominantly during replication (13). The numbers of repeat motifs differ in a population, thereby creating multiple alleles at an MS locus. MS loci are amplified by PCR using fluorescently labeled forward and unlabeled reverse primers. The dye-labeled products are separated by size using automated electrophoresis and identified by fluorescence detection. We previously designed a multiplex PCR assay with 5 MS markers that were able to reach the typing level but not the fingerprinting level of discrimination between strains (4). Here we developed an easy-to-use and rapid genotyping method which aimed to ensure both levels of genetic discrimination of *T. gondii* isolates in a single multiplex PCR assay using 15 MS markers located on 11 different chromosomes of *T. gondii*. In MS sequences with dinucleotide motifs, those with $[TG/AC]_n$ and $[TC/AG]_n$ repeats are known to exhibit lower mutability than those with $[TA/AT]_n$ repeats (13). Eight MS markers with a repeat motif consisting of $[TG/AC]_n$ or $[TC/AG]_n$ were used as typing markers: five markers (*TUB2*, *W35*, *TgM-A*, *B18*, and *B17*) were described in our previous report of a multiplex assay (4), *M33* was developed by others (6), and two markers (*IV.1* and *XI.1*) were designed for this study. Seven MS markers with a repeat motif consisting of $[TA/AT]_n$ were used as fingerprinting markers: five markers (*N60*, *N82*, *AA*, *N61*, and *N83*) were described by us in a previous study (2) whereas *M48* and *M102* were developed by others (6). For some markers, primers sequences were redesigned to allow PCR amplification and allele size discrimination in the multiplex assay. Chromosome locations and primer sequences for the 15 MS markers are listed in Table 1. Primers were synthesized by Applied Biosystems (Courtabœuf, France). PCR was carried out in a 25-μl reaction mixture consisting of 12.5 μl of 2× Qiagen multiplex PCR Master Mix (Qiagen, France) and 5 pmol each primer. The volumes of DNA template were 1 μl for DNA extracted from tachyzoites in cell culture or mouse ascitic fluid, 3 μl for DNA extracted from bradyzoites in mouse brain, and 5 μl for DNA extracted directly from clinical samples. Cycling conditions

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TABLE 1. Microsatellite markers and PCR primers used for the multiplex PCR assay

| Marker | Chromosome (position)[a] | Repeat motif(s) | Primer sequence[b] | Size range (bp) |
|---|---|---|---|---|
| *TUB2* | IX (974608 to 974896) | $[TG/AC]_n$ | (F) 5′ 6-FAM-GTCCGGGTGTTCCTACAAAA 3′<br>(R) 5′ TTGGCCAAAGACGAAGTTGT 3′ | 287–291 |
| *W35* | II (633241 to 633482) | $[TC/AG]_n$, $[TG/AC]_n$ | (F) 5′ HEX-GGTTCACTGGATCTTCTCCAA 3′<br>(R) 5′ AATGAACGTCGCTTGTTTCC 3′ | 242–248 |
| *TgM-A* | X (4824879 to 4825083) | $[TG/AC]_n$ | (F) 5′ HEX-GGCGTCGACATGAGTTTCTC 3′<br>(R) 5′ TGGGCATGTAAATGTAGAGATG 3′ | 203–211 |
| *B18* | VIIa (2921536 to 2921693) | $[TG/AC]_n$ | (F) 5′ 6-FAM-TGGTCTTCACCCTTTCATCC 3′<br>(R) 5′ AGGGATAAGTTTCTTCACAACGA 3′ | 156–170 |
| *B17* | XII (6474746 to 6475079) | $[TC/AG]_n$ | (F) 5′ HEX-AACAGACACCCGATGCCTAC 3′<br>(R) 5′ GGCAACAGGAGGTAGAGGAG 3′ | 334–366 |
| *M33* | IV (672591 to 672760) | $[TC/AG]_n$ | (F) 5′ 6-FAM- TACGCTTCGCATTGTACCAG 3′<br>(R) 5′ TCTTTTCTCCCCTTCGCTCT 3′ | 165–173 |
| *IV.1* | IV (742419 to 742693) | $[TG/AC]_n$ | (F) 5′ HEX-GAAGTTCGGCCTGTTCCTC 3′<br>(R) 5′ TCTGCCTGGAAAAGGAAAGA 3′ | 272–282 |
| *XI.1* | XI (189702 to 190058) | $[TG/AC]_n$ | (F) 5′ 6-FAM-GCGTGTGACGAGTTCTGAAA 3′<br>(R) 5′ AAGTCCCCTGAAAAGCCAAT 3′ | 354–362 |
| *M48* | Ia (332951 to 333166) | $[TA/AT]_n$ | (F) 5′ 6-FAM-AACATGTCGCGTAAGATTCG 3′<br>(R) 5′ CTCTTCACTGAGCGCCTTTC 3′ | 209–243 |
| *M102* | VIIa (3093491 to 3093664) | $[TA/AT]_n$ | (F) 5′ NED-CAGTCCAGGCATACCTCACC 3′<br>(R) 5′ CAATCCCAAAATCCCAAACC 3′ | 164–196 |
| *N60* | Ib (1766079 to 1766221) | $[TA/AT]_n$ | (F) 5′ NED-GAATCGTCGAGGTGCTATCC 3′<br>(R) 5′ AACGGTTGACCTGTGGCGAGT 3′ | 132–157 |
| *N82* | XII (1621472 to 1621585) | $[TA/AT]_n$ | (F) 5′ HEX-TGCGTGCTTGTCAGAGTTC 3′<br>(R) 5′ GCGTCCTTGACATGCACAT 3′ | 105–145 |
| *AA* | VIII (5836880 to 5837144) | $[TA/AT]_n$ | (F) 5′ NED-GATGTCCGGTCAATTTTGCT 3′<br>(R) 5′ GACGGGAAGGACAGAAACAC 3′ | 251–332 |
| *N61* | VIIb (4217145 to 4217238) | $[TA/AT]_n$ | (F) 5′ 6-FAM-ATCGGCGGTGGTTGTAGAT 3′<br>(R) 5′ CCTGATGTTGATGTAAGGATGC 3′ | 79–123 |
| *N83* | X (1772898 to 1773209) | $[TA/AT]_n$ | (F) 5′ 6-FAM-ATGGGTGAACAGCGTAGACA 3′<br>(R) 5′ GCAGGACGAAGAGGATGAGA 3′ | 306–338 |

[a] Based on position within each chromosome of strain ME49 as given in the Toxo DB website (http://toxodb.org).

[b] (F), forward primer; (R), reverse primer. In each pair of primers, the forward primer was labeled at the 5′ end with flurorescein: 6-carboxyfluorescein (6-FAM) for MS *TUB2*, *XI.1*, *B18*, *N83*, *N61*, *M33*, and *M48*, hexachlorofluorescein (HEX) for MS *TgM-A*, *B17*, *N82*, *W35*, and *IV.1*, and 2,7′,8′-benzo-5′-fluoro-2′,4,7-trichloro-5-carboxyfluorescein (NED) for MS *AA*, *N60*, and *M102*.

were 15 min at 95°C; 30 s at 94°C, 3 min at 61°C, and 30 s at 72°C (35 cycles); and 30 min at 60°C. PCR products were diluted in deionized formamide as follows: 1/20 for DNA extracted from tachyzoites in cell culture or mouse ascitic fluid, 1/10 for DNA extracted from bradyzoites in mouse brain, and 1/2 for DNA extracted directly from clinical samples. One microliter of each diluted PCR product was mixed with 0.5 μl of a dye-labeled size standard (ROX 500; Applied Biosystems) and 23.5 μl of deionized formamide. This mixture was denatured at 95°C for 5 min and then electrophoresed using an automatic sequencer (ABI PRISM 3130xl; Applied Biosystems). The sizes of the alleles in base pairs were estimated using GeneMapper analysis software (version 4.0; Applied Biosystems) (Fig. 1).

We used the 15-MS multiplex assay to genotype 26 reference isolates that had been extensively characterized with other sets of markers. These strains have been classified either into the three main clonal lineages (types I, II, and III as classified by PCR-RFLP markers, corresponding to haplogroups 1, 2, and 3 as classified by intron sequencing, respectively) or into atypical genotypes by PCR-RFLP markers corresponding to haplogroups 4 to 11 by intron sequencing (Table 2). The genotyping results of the 15-MS multiplex assay showed that the 8 typing markers (*TUB2*, *W35*, *TgM-A*, *B18*, *B17*, *M33*, *IV.1*, and *XI.1*)

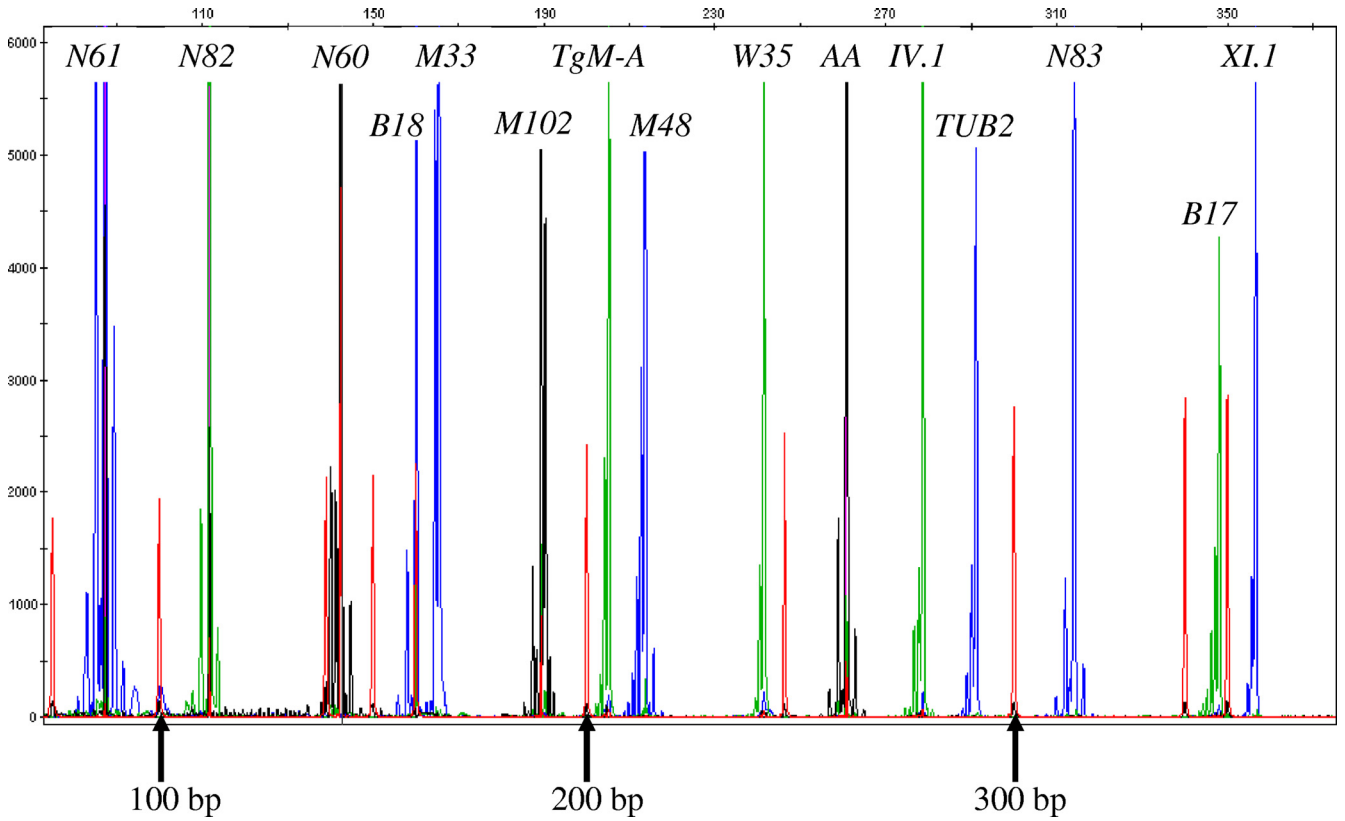


FIG. 1. Electrophoresis of the 15-microsatellite multiplex PCR amplification products from strain P89 by the use of an automatic sequencer and GeneMapper software. The *x* axis indicates a size fragment scale (in base pairs). The *y* axis indicates a peak height scale corresponding to the fluorescence intensities of detected peaks.

could readily distinguish clonal types I, II, and III from all the atypical genotypes (Table 2). In each of clonal lineages I to III or haplogroups 1 to 3, representative strains had uniform typing patterns, except for one for lineage II, where allele 244 at marker *W35* was identified for isolate BOU. Such variants within clonal lineage II have also been previously determined at the *Apico* locus with PCR-RFLP markers (17). Atypical isolates were characterized by a different mix of alleles of haplogroups 1 to 3 and by additional atypical alleles at some loci. The detection of atypical alleles by the MS markers underscores the ability of these markers to capture the genetic diversity of highly divergent strains such as South American strains (1, 14, 15). The 7 fingerprinting markers (*M48*, *M102*, *N60*, *N82*, *AA*, *N61*, and *N83*) provided enhanced genetic resolution, beyond the typing level, in distinguishing closely related isolates within one clonal lineage or haplogroup (Table 2). The Simpson's index of diversity (*D*) (2) was maximal ($D = 1$), since all reference isolates had a unique genotype as classified with the 15 MS markers.

The association of the 15 MS markers with different mutability patterns enabled two different levels of genetic resolution for differentiating *T. gondii* strains at the typing level (types I, II, and III versus atypical strains) and at the fingerprinting level (closely related isolates within a clonal lineage). Multilocus sequence-based typing is the best tool for estimating the true rate of DNA polymorphism in phylogenetic studies, but the increased cost and investment of time makes that method unsuitable for genetic screening of a large number of isolates. PCR-RFLP markers are preferred tools for the latter purpose, but their low resolution level makes them inadequate for distinguishing genetically closely related strains. The 15-MS multiplex assay is therefore the best available tool to reveal whether two isolates are genetically identical or different, i.e., to identify a common source of infection in an outbreak, a laboratory contamination, and mixed infections. The limitations of this assay are the requirement for the availability of an automated sequencer and the sensitivity of the method, which has been estimated by testing serial dilutions of RH strain tachyzoites to be between 50 to 100 *T. gondii* genome equivalents per 5 μl of DNA sample. Testing this method on samples with lower DNA concentrations is not recommended because of incomplete amplification of markers or difficulties in interpreting the results due to overlaps of microsatellite peaks with nonspecific PCR products. Compared with other genotyping methods, the sensitivity appears similar to that reported with intron sequencing. PCR-RFLP analysis can detect as few as 10 *T. gondii* genome equivalents per PCR, but such a threshold of detection is reached only by nested PCR assays (16). We did not develop a nested PCR assay for improving the sensitivity of our 15-MS multiplex assay since, in our experience, double-round PCR amplification using this method represents too

TABLE 2. Genotyping results of 26 reference strains with 15 MS markers in a single multiplex PCR assay

| Type (haplogroup)[a] | Isolate[b] | Origin (host)[c] | Microsatellite marker | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *TUB2* | *W35* | *TgM-A* | *B18* | *B17* | *M33* | *IV.1* | *XI.1* | *M48* | *M102* | *N60* | *N82* | *AA* | *N61* | *N83* |
| I (1) | P | France (H) | 291 | 248 | 209 | 160 | 342 | 169 | 274 | 358 | 209 | 166 | 145 | 121 | 267 | 87 | 308 |
| I (1) | CT1 | USA (cow) | 291 | 248 | 209 | 160 | 342 | 169 | 274 | 358 | 209 | 168 | 145 | 119 | 265 | 87 | 306 |
| I (1) | GIL | France (H) | 291 | 248 | 209 | 160 | 342 | 169 | 274 | 358 | 209 | 166 | 147 | 119 | 265 | 87 | 306 |
| I (1) | BK | Netherlands (H) | 291 | 248 | 209 | 160 | 342 | 169 | 274 | 358 | 209 | 166 | 145 | 119 | 265 | 87 | 316 |
| II (2) | NTE | Germany (H) | 289 | 242 | 207 | 158 | 336 | 169 | 274 | 356 | 221 | 176 | 140 | 113 | 269 | 103 | 310 |
| II (2) | PTG | USA (sheep) | 289 | 242 | 207 | 158 | 336 | 169 | 274 | 356 | 215 | 174 | 142 | 111 | 265 | 91 | 310 |
| II (2) | BOU | France (H) | 289 | 244 | 207 | 158 | 336 | 169 | 274 | 356 | 213 | 178 | 140 | 113 | 259 | 99 | 310 |
| III (3) | CTG | USA (cat) | 289 | 242 | 205 | 160 | 336 | 165 | 278 | 356 | 215 | 190 | 147 | 111 | 269 | 89 | 312 |
| III (3) | NED | France (H) | 289 | 242 | 205 | 160 | 336 | 165 | 278 | 356 | 209 | 190 | 147 | 111 | 267 | 91 | 312 |
| III (3) | M7741 | USA (sheep) | 289 | 242 | 205 | 160 | 336 | 165 | 278 | 356 | 215 | 190 | 147 | 111 | 267 | 91 | 312 |
| III (3) | VEG | USA (H) | 289 | 242 | 205 | 160 | 336 | 165 | 278 | 356 | 213 | 188 | 153 | 111 | 267 | 89 | 312 |
| Atypical (4) | MAS | France (H) | 291 | 242 | 205 | 162 | 362 | 169 | 272 | 358 | 221 | 166 | 142 | 111 | 332 | 95 | 338 |
| Atypical (4) | CASTELLS | Uruguay (sheep) | 287 | 242 | 207 | 158 | 358 | 169 | 274 | 356 | 239 | 164 | 138 | 109 | 283 | 87 | 324 |
| Atypical (4) | TgCatBr1 | Brazil (cat) | 289 | 242 | 205 | 160 | 342 | 165 | 278 | 358 | 233 | 164 | 147 | 111 | 316 | 89 | 308 |
| Atypical (5) | GUY-KOE | Fr. Guiana (H) | 289 | 246 | 203 | 160 | 337 | 165 | 274 | 356 | 209 | 172 | 136 | 111 | 251 | 109 | 310 |
| Atypical (5) | GUY-MAT | Fr. Guiana (H) | 291 | 242 | 203 | 160 | 339 | 165 | 272 | 358 | 221 | 174 | 138 | 107 | 277 | 95 | 312 |
| Atypical (5) | RUB | Fr. Guiana (H) | 289 | 242 | 205 | 170 | 360 | 167 | 274 | 356 | 223 | 190 | 142 | 109 | 259 | 85 | 312 |
| Atypical (6) | GPHT | France (H) | 291 | 248 | 205 | 160 | 342 | 165 | 274 | 354 | 229 | 166 | 147 | 111 | 283 | 91 | 306 |
| Atypical (6) | BOF | Belgium (H) | 291 | 248 | 205 | 160 | 342 | 165 | 274 | 354 | 227 | 166 | 147 | 111 | 273 | 89 | 306 |
| Atypical (7) | CAST | USA (H) | 291 | 242 | 205 | 158 | 342 | 167 | 276 | 356 | 211 | 168 | 147 | 119 | 279 | 87 | 306 |
| Atypical (8) | TgCatBr5 | Brazil (cat) | 291 | 242 | 205 | 160 | 362 | 165 | 278 | 356 | 237 | 174 | 140 | 111 | 265 | 89 | 314 |
| Atypical (9) | P89 | USA (pig) | 291 | 242 | 205 | 160 | 348 | 165 | 278 | 356 | 213 | 190 | 142 | 111 | 261 | 87 | 314 |
| Atypical (9) | TgCatBr3 | Brazil (cat) | 289 | 242 | 205 | 160 | 348 | 165 | 278 | 356 | 213 | 190 | 142 | 111 | 263 | 113 | 312 |
| Atypical (10) | VAND | Fr. Guiana (H) | 291 | 242 | 203 | 162 | 344 | 167 | 276 | 356 | 217 | 170 | 142 | 113 | 277 | 91 | 308 |
| Atypical (10) | GUY-DOS | Fr. Guiana (H) | 289 | 246 | 203 | 160 | 344 | 167 | 272 | 356 | 229 | 176 | 142 | 113 | 263 | 85 | 312 |
| Atypical (11) | COUGAR | Canada (cougar) | 289 | 242 | 205 | 158 | 336 | 169 | 274 | 354 | 219 | 174 | 151 | 119 | 259 | 79 | 332 |

[a] The types were defined by analysis of single nucleotide polymorphisms at 5 or 10 PCR-RFLP markers (12, 17); the haplogroups were defined by analysis of sequence polymorphisms within 8 introns of five unlinked loci (14).

[b] Strain P is also known as strain PT; strain PTG is a clone of the ME49 strain; strain CTG is also known as strain CEP or C; strains GUY-KOE, GUY-MAT, and GUY-DOS are also known as strains GUY-2002-KOE, GUY-2002-MAT, and GUY-2001-DOS, respectively; strain P89 is also known as strain TgPgUs15; strain COUGAR is also known as strain TgCgCa1 or COUG.

[c] Fr. Guiana, French Guiana; H, human; UK, United Kingdom; USA, United States.

high a risk of PCR contamination and often leads to misleading results.

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